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(54) Title: METHOD OF TREATING SEPSIS

(57) Abstract

The invention relates to the method of preventing and treating sepsis using chemokines selected from mature or modified KC [SEQ ID NO: 1], groα [SEQ ID NO:2], groβ [SEQ ID NO:3] or groγ [SEQ ID NO:4] or multimers thereof, alone or in conjunction with an anti-infective agent.

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# METHOD OF TREATING SEPSIS

## Field of Invention

This invention relates to the method of preventing and treating sepsis using certain chemokines alone or in conjunction with an anti-infective agent.

## Background of Invention

Sepsis, as used herein, is broadly defined to mean situations when the invasion of a host by a microbial agent is associated with the clinical manifestations of infection including but not limited to: (1) temperature >38°C or <36°C; (2) heart rate >90 beats per minute; (3) respiratory rate >20 breaths per minute or $PaCO_2$ <32 mm Hg; (4) white blood cell count >12,000/cu mm, <4,000/cu mm, or >10% immature (band) forms; (5) organ dysfunction, hypoperfusion, or hypotension. Hypoperfusion and perfusion abnormalities may include, but are not limited to lactic acidosis, oliguria, or an acute alteration in mental states. (Chest 1992; 101: 1644-1566)

Sepsis can occur in hospitalized patients having underlying diseases or conditions that render them susceptible to bloodstream invasion or in burn, trauma or surgical patents. In many cases of sepsis, the predominant pathogen is *Escherichia coli*, followed by other Gram-negative bacteria such as the *Klebsiella-Enterobacter-Serratia* group and then *Pseudomonas*. Although comprising a somewhat smaller percentage of infection, Gram-positive microbes such as *Staphylococcus* and systemic viral and fungal infections are included by the term sepsis as used herein. The genitourinary tract is the most common site of infection, the gastrointestinal tract and respiratory tract being the next most frequent sources of sepsis. Other common foci are wound, burn, and pelvic infections and infected intravenous catheters.

A serious consequence of bacterial sepsis often is septic shock. Septic shock is characterized by inadequate tissue perfusion, leading to insufficient oxygen supply to tissues, hypotension and oliguria.

Septic shock occurs because bacterial products react with cells and components of the coagulation, complement, fibrinolytic and bradykinin systems to release proteases which injure cells and alter blood flow, especially in the capillaries.

Microorganisms frequently activate the classical complement pathway, and endotoxin activates the alternative pathway. Complement activation, leukotriene generation and the direct effects of bacterial products on neutrophils lead to accumulation

of these inflammatory cells in the lungs, release of their proteolytic enzymes and toxic oxygen radicals which damage the pulmonary endothelium and initiate the adult respiratory distress syndrome ("ARDS"). ARDS is a major cause of death in patients with septic shock and is characterized by pulmonary congestion, granulocyte aggregation, hemorrhage and capillary thrombi.

Septic shock is a major cause of death in intensive care units. There are an estimated 200,000 cases per year of septic shock in the United States, and despite advances in technology (i.e., respiratory support) and antibiotic therapy, the mortality rate for septic shock remains in excess of 40%. In fact, mortality for established septic shock has decreased very little since the comprehensive description by Waisbren (Arch. Intern. Med. 88:467-488 (1951)). Although effective antibiotics are available, and there is an increased awareness of the septic shock syndrome, the incidence of septic shock over the last several decades has actually increased. With the appreciation that antimicrobial agents have failed to completely abrogate septic mortality, it is clear that other agents must be developed to be used alone or in conjunction with antimicrobials in order to rectify the deficiencies of current established therapy.

## Summary of the Invention

This invention relates to a method of preventing or treating sepsis comprising administering to a human or non-human animal in need thereof an effective amount of a protein derived from a chemokine selected from KC, gro-a, groß, and groγ. Most preferably, the chemokines used in the method of the invention include modified KC [amino acids 5-72 of the full length protein, SEQ ID NO: 1], modified human groß [amino acids 5-73 of the full length protein, SEQ ID NO: 3] or modified human groγ [amino acids 5-73 of the full length protein, SEQ ID NO: 4] or multimers thereof. Alternatively, the mature chemokines may be utilized in the method of the invention.

The method of the invention may be performed alone, or in conjunction with administration of an anti-infective agent.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

**Detailed Description of the Invention**

It is the object of this invention to provide a new method of treatment of sepsis comprising administering to an animal in need thereof, including humans, an effective amount of a chemokine. The chemokines useful in the method of the invention include mature KC [SEQ ID NO:1], groα [SEQ ID NO:2], groß [SEQ ID NO:3], groγ [SEQ ID NO:4], or the modified and multimeric proteins derived therefrom, which are described in detail in International Patent Application, Publication No. WO94/29341, incorporated by reference herein. Particularly desirable are the modified KC [amino acids 5-72 of SEQ ID NO:2], modified groß [amino acids 5-73 of SEQ ID NO:3], modified groγ [amino acids 5-73 of SEQ ID NO:4], and a dimeric modified groß [amino acids 5-73 of SEQ ID NO:3].

Although these chemokines have been previously described, their use in prevention and treatment of sepsis has not been reported. It has now been discovered that mature KC [SEQ ID NO: 1], human groα [SEQ ID NO:2], human groß [SEQ ID NO: 3] or human groγ [SEQ ID NO: 4], and, particularly the modified and multimeric chemokines derived therefrom significantly increase the survival of animals challenged with lethal sepsis causing organisms. Treatment with a medicament or the compound of this invention, alone or in combination with an anti-infective agent prior to contemplated thoracic or abdominal surgery would be useful in reducing the likelihood of post-operative sepsis. It may also be used post-operatively for the treatment of sepsis caused by a variety of reasons as outlined previously.

As stated above, the proteins useful in preparing medicaments and in the methods of the invention include the mature chemokines, modified chemokines, and multimers thereof.

The term "mature chemokines" also known as "intercrines", as used herein defines the proteins conventionally referred to in the art as KC, *groα*, *groß*, and *groγ*. For convenience, the amino acid sequences of the murine protein KC which contains 72 residues is provided in SEQ ID NO:1. These sequences are available from Genbank, accession number J04596. The sequences of the human protein *groα* (aa 1-73) are provided in SEQ ID NO:2. The sequences of the human protein *groß* (amino acids 1-73) are provided in SEQ ID NO: 3. The sequences of the human protein *groγ* are provided in SEQ ID NO:4. The cDNA and amino acid sequences of *groγ* are also provided in International Patent Application, Publication No. WO 92/00326 (Jan. 9, 1992). These *groγ*

sequences have further been published in International Patent Application, Publication No. WO 94/29341 (December 22, 1994), which is incorporated by reference herein.

The term "modified chemokines" is defined as in the above-referenced International Application. The modified chemokines are derived from KC, *groß*, *groα*, and *groγ*, more preferably from *groß*, *groα*, and *groγ*, and most preferably from *groß*. The modified chemokines include desamino proteins characterized by the elimination of between about 2 to about 8 amino acids at the amino terminus of the mature protein. Most preferably, the modified chemokines are characterized by removal of the first 4 amino acids at the amino- (N-) terminus. Optionally, particularly when expressed recombinantly, the desamino chemokines useful in this invention may contain an inserted N-terminal Met. The N-terminal methionine which is inserted into the protein for expression purposes, may be cleaved, either during the processing of the protein by a host cell or synthetically, using known techniques. Alternatively, if so desired, this amino acid may be cleaved through enzyme digestion or other known means. Particularly desirable modified chemokines include modified KC [amino acids 5 - 72 of SEQ ID NO: 1], modified human groß [amino acids 5-73 of SEQ ID NO: 3] and modified human groγ [amino acids 5-73 of SEQ ID NO: 4].

Also included by the term modified chemokine are other analogs or derivatives of KC, groα, groß, or groγ which share the biological activity of the mature protein. As defined herein, such analogs and derivatives include modified proteins also characterized by alterations made in the known amino sequence of the proteins, e.g., the proteins provided in SEQ ID NOS: 1-4. Such analogs are characterized by having an amino acid sequence differing from that of the mature protein by 8 or fewer amino acid residues, and preferably by about 5 or fewer residues. It may be preferred that any differences in the amino acid sequences of the proteins involve only conservative amino acid substitutions. Conservative amino acid substitutions occur when an amino acid has substantially the same charge as the amino acid for which it is substituted and the substitution has no significant effect on the local conformation of the protein or its biological activity. Alternatively, changes such as the introduction of a certain amino acid in the sequence which may alter the stability of the protein, or permit it to be expressed in a desired host cell may be preferred. Another characteristic of these modified proteins may be enhanced biological activity in comparison to the mature protein.

By the term "multimeric protein" or "multimer" is meant herein multimeric forms of the mature and/or modified proteins useful in this invention, e.g., dimers, trimers, tetramers and other aggregated forms. Such multimeric forms can be prepared by synthesis or recombinant expression and can contain chemokines produced by a combination of synthetic and recombinant techniques as detailed below. Multimers may form naturally upon expression or may be constructed into such multiple forms. Multimeric chemokines may include multimers of the same modified chemokine. Another multimer may be formed by the aggregation of different modified proteins. Still another multimer is formed by the aggregation of a modified chemokine of this invention and a known, mature chemokine. Preferably, a dimer or multimer useful in the invention would contain at least one desamino chemokine protein and at least one other chemokine or other protein characterized by having the same type of biological activity. This other protein may be an additional desamino chemokine, or another known protein. In one particularly desirable embodiment, the method of the invention utilizes a dimeric truncated groß protein [amino acids 5-73 of SEQ ID NO:3], which is described in more detail below.

Desirably, the chemokines useful in the method of the invention are used in the preparation of medicaments and/or are useful in the form of a pharmaceutical composition. Thus, the chemokines can be formulated into pharmaceutical compositions and administered in the same manner as described in, e.g., International Patent Applications, Publication No. WO 90/02762 (Mar. 22, 1990) and Publication No. WO94/29341 (Dec. 22, 1994).

These medicaments or pharmaceutical compositions useful in the method of the invention for preventing or treating sepsis contain an effective amount of a mature, modified or multimeric chemokine protein derived from KC [SEQ ID NO: 1], human gro-a [SEQ ID NO: 2], human groß [SEQ ID NO: 3], and human groγ [SEQ ID NO: 4] which is administered to an animal in need thereof. Particularly desired embodiments utilize the modified chemokines, or multimers thereof These chemokine compositions may be administered alone or in combination with administration of other anti-infective agents.

Thus, a pharmaceutical composition is prepared using one or more of proteins derived from the KC [SEQ ID NO:1], groα [SEQ ID NO:2], groß [SEQ ID NO:3] or groγ [SEQ ID NO:4] proteins. Suitable pharmaceutical carriers are well known to those of skill in the art and may be readily selected. Currently, the preferred carrier is saline. Optionally, the pharmaceutical compositions of the invention may contain other active ingredients or be administered in conjunction with other therapeutics. For example, the compositions of

the invention are particularly well suited for administration in conjunction with anti-infective agents.

Suitable anti-infective agents include, without limitation, anti-microbial agents routinely used for the treatment of sepsis such as amino-glycosides (such as amikacin, tobramycin, netilmicin, and gentamicin), cephalosporins such as ceftazidime, related beta-lactam agents such as maxalactam, carbopenems such as imipenem, monobactam agents such as aztreonam; ampicillin and broad-spectrum penicillins, (e.g., penicillinase-resistant penicillins, ureidopenicillins or antipseudomonal penicillin or Augmentin) that are active against *P. aeruginosa*, *Enterobacter* species, indole-positive *Proteus* species, and *Serratia*. Also included within the definition of anti-infective agents are antifungal agents, amphotericin and the like as well as anti-viral agents such as famvir and acyclovir.

The chemokines described herein are useful in the treatment and prevention of sepsis in humans and other animals such as dairy cattle, horses, calves or poultry. To effectively treat a human or other animal a mature, modified or multimeric KC [SEQ ID NO: 1], groα [SEQ ID NO: 2], groß [SEQ ID NO:3] or human groγ [SEQ ID NO: 4] or their multimers (e.g., a dimeric, truncated groß, amino acids 5-73 of SEQ ID NO:3) may be administered by injection in the dose range of about 10 to about 10,000 fg/kg/dose, or orally in the dose range of about 10 to about 10,000 fg/kg body weight per dose; if administered by infusion or similar techniques, the dose may be in the range of about 10 to about 10,000 fg/kg/dose; if administered subcutaneously the dose may be in the range of about 10 to about 10,000 fg/kg/dose.

Depending on the patient's condition, the compounds of this invention can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, the compound is administered to a patient already suffering from a disease in an amount sufficient to cure or at least partially arrest the disease and its complications. It may be given at any time after surgery, preferably prior to 24 hours after surgery. In prophylactic applications, a composition containing mature, modified or multimeric KC [SEQ ID NO: 1], groα [SEQ ID NO: 2], groß [SEQ ID NO:3] or groγ [SEQ ID NO: 4] or a multimer thereof, is administered to a patient not already in a disease state to enhance the patient's resistance. It may be given one day or one week prior to surgery, preferably one to two days prior to surgery. It may be administered parenterally or orally.

Single or multiple administrations of the compounds can be carried out with dose levels and pattern being selected by the treating physician. In any event, a quantity of the

compounds of the invention sufficient to effectively treat the patient should be administered.

The chemokines useful in the methods of this invention, may also be administered in conjunction with a separately administered conventional anti-infective as disclosed herein above, such a gentamicin, augmentin or ceftazidime. The particular anti-infective chosen should be one to which the infective organism is susceptible and is selected or modified during therapy as the infecting microorganism is more particularly identified.

Additionally, various adjunctive agents in the treatment of septic shock also may be useful in combination with the components of this invention. They include sympathomimetic amines (vasopressors) such as norepinephrine, epinephrine, isoproterenol, dopamine, and dobutamine; anti-inflammatory agents such as methylprednisolone anti-inflammatory agents such as indomethacin and phenylbutazone; and corticosteroids such as betamethasone, hydrocortisone, methylprednisolone, or dexamethasone; anti-coagulants such as heparin, anti-thrombin III or coumarin type drugs for certain conditions and schedules; diuretics such as furosemide or ethacrynic acid; and antagonist of opiates and beta-endorphins such as naloxone; an antagonist of tumor necrosis factor or of interleukin-1; phenothiazines; anti-histamines; glucagon; a-adrenergic blocking agents, vasodilators; plasma expanders; packed red blood cells; platelets; cryoprecipitates; fresh frozen plasma; bacterial permeability protein; clindamycin; and antibodies to (lipid A), the J5 mutant of *E. coli* or to endotoxin core glycolipids. Methods for preparing such antibodies are described widely in the literature.

One of the most important aspects in the treatment of the clinical septic shock syndrome is its apparently intractable resistance to the effects of a variety of highly potent antimicrobial agents. Despite the development of newer antimicrobial agents, the overall incidence of clinical sepsis has increased, and mortality remains unacceptably high, often approaching 60% of diagnosed patients. The discovery of the increased survival with the treatment of the full length, modified and multimeric KC [SEQ ID NO: 1], groα [SEQ ID NO: 2], groß [SEQ ID NO:3], or groγ [SEQ ID NO: 4] both prophylactically and after infection provides a new and useful therapy of sepsis.

The biological activity of modified KC [SEQ ID NO: 1], modified human groß [SEQ ID NO: 3], modified human groγ [SEQ ID NO: 4], and a dimeric modified human groγ are demonstrated by the following assays. These examples illustrate the preferred methods of the invention. These examples do not limit the scope of the invention.

**Rats.** Male Fischer 344 rats obtained from Taconic farms weighing 200 to 250 g. were utilized. The rats were housed 2 per cage in standard plastic caging and fed lab chow and water ad libitum.

Modified KC [SEQ ID NO: 1], modified human groß [SEQ ID NO: 2] or modified human groγ [SEQ ID NO: 3] or multimers thereof, was prepared in *E. coli* by the method given in Example 1. The compound was dissolved in DPBS containing 0.5 % heat inactivated autologous normal rat serum. The animals were dosed intraperitoneally with KC 24 hours and 2 hours before infection. Control animals were dosed with dilution buffer on the same schedule. Starting two hours after infection the rats were treated twice daily with subcutaneous gentamicin.

**E. coli.** A clinical isolate of *E. coli* isolated from sputum was utilized. The organisms were tested for antibiotic sensitivity by the disc-agar diffusion technique and found to be sensitive to gentamicin, ampicillin, cephalothin, chloramphenicol, kanamycin, tetracycline, trimethoprin/sulfamethoxazole and resistant to penicillin G, erythromycin, and vancomycin. The organism was animal passed in mice and subsequently recovered and plated onto MacConkey's agar. The reisolated organisms were grown overnight in brain-heart infusion broth, and then stored frozen at -70°C. The inoculate the fibrin clot, organisms from thawed stocks were inoculated into brainheart infusion broth and incubated overnight on a rotary shaker (120 rpm) an 37°C. The *E. coli* was harvested by centrifugation, washed 3X and finally resuspended in normal saline. The number or organisms was quantified by turbidimentry, and the concentration adjusted with normal saline. All inoculum sizes were based on viable counts determined by scoring colony forming units on MacConkeys agar.

**Fibrin Clot.** The *E. coli* infected fibrin clots were made from a 1% solution of bovine fibrinogen (Type 1-S, Sigma) in sterile saline. The clot was formed by adding sequentially human thrombin (Hanna Pharma.) bacteria, and fibrinogen solution to 24 well plastic plates. Bacterial numbers of 2.0 to 3.0 x $10^9$ were used in inoculate the fibrin clots. The resulting mixture was then incubated at room temperature for 30 minutes before implantation.

**Animal Model.** The rats were anesthetized with ketamine/xylazine (40 mg/kg/5 mg/kg) after which the abdominal surfaced were shaved and a midline laparotomy performed. Bacterial peritonitis was induced by implanting a fibrin-thrombin clot containing *E. coli* into the abdominal cavity. After implantation the muscle layers were closed with 4-0 silk suture, and the wound closed with surgical staples. The animals were closely observed, any animals obviously moribund were euthanized.

**Gentamicin.** Rats were treated subcutaneously with gentamicin sulfate (Elkins-Sinn, NJ) 5 mg/kg twice a day for five days.

**Statistics.** All continuously variable data are expressed as the percent survival from several pooled studies. The Fisher's Exact test was used to determine the statistical significance of the differences between the survival rates at 14 days. The differences between the groups were considered statistically significant at $p<0.05$.

**Example 1 - Production of Truncated KC and GROß**

A. *Expression of recombinant truncated KC and truncated groß.*

When truncated murine KC (amino acids 5-72 of SEQ ID NO:1) and human groß (amino acids 5-73 of SEQ ID NO:3) were expressed intracellularly in *E. coli*, the KC (amino acids 5-72 of SEQ ID NO:1) retained the initiator Met. In order to produce the authentic N-terminal recombinant proteins, a specific cleavable tag was engineered at the N-terminus of truncated KC (amino acids 5-72 of SEQ ID NO:1). The coding sequences of truncated murine KC and truncated human groß (amino acids 5-73 of SEQ ID NO:3) were each amplified by polymerase chain reaction (PCR) from plasmids containing complimentary DNA sequences using both a forward primer encoding an NdeI site and a reverse primer containing an XbaI site. For truncated KC (amino acids 5-72 of SEQ ID NO:3), a defined epitope tag (DET) site and an enterokinase cleavage site were also used. These PCR fragments were subcloned into the *E. coli* LPIL-dependent expression vector pEAKn (pSKF301 derivative) between NdeI and XbaI sites. Each polypeptide was expressed by chemical induction of the LPL promoter in a lysogenic strain of E. coli containing the wild type (ind+) repressor gene (cI+) AR120.

B. *Purification and refolding of truncated groß (amino acids 5-73 of SEQ ID NO:3)*

*E. coli* cell pellet was lysed in pH 6.0 buffer containing 20 mM dithiothreitol (DTT) to avoid the nonspecific air oxidation. The majority of truncated groß was in the insoluble lysate pellet which was solubilized in 2 M GdnHCl, pH 8.0 buffer containing 20mM DTT. The solubilized truncated groß was dialyzed against pH 6.0 buffer containing 2 mM EDTA. The majority of *E. coli* proteins were precipitated during dialysis while truncated groß stayed in solution as a monomeric form at >95 % purity. The truncated groß solution was adjusted to pH 8.5, stirred overnight for air oxidation. The refolded truncated groß solution was adjusted to 0.1 % TFA solution, and applied to Ultrasphere C18 (Beckman) column to separate monomeric form from dimeric form. Each form was pooled separately, evaporated to remove acetonitrile, concentrated, dialyzed against PBS and stored at -70°.

C. *Purification of truncated KC (amino acids 5-72 of SEQ ID NO:1)*

DET-DDDDK chemokines were purified and refolded as described for truncated groß. The refolded DET-DDDDK chemokines were digested with enterokinase to remove the N-terminal DET-DDDDK and the undigested molecules were removed using anti DET Mab column. The digested molecules were further purified using C18 RP-HPLC as described above.

D. *Characterization*

N-terminal sequencing and MALD-MS for molecular weight were performed and confirmed that the molecules are intact from N-terminus to C-terminus, either monomeric or dimeric form. Concentration of each chemokine was determined by amino acid analysis and endotoxin level of each prep was <0.05 U/ml.

**Example 2 - Production of Truncated GROß Dimer**

A. *Cell lysis*

*E. coli* LW cells, 400 g, were lysed in 4 liters of lysis buffer containing 50 mM sodium citrate pH 6.0, 40 mM NaCl, 2 mM EDTA, 5% glycerol, 0.05% Tween 80, 0.2 mM PMSF, 1 mg/ml each of leupeptin and pepstatin A, by two passages through a Microfluidics (model M110Y) homogenizer at 11,000 psi. The cell lysate was centrifuged at 17,000 g (one hour at 4°C) and the supernatant was discarded.

B. *Solubilization and Refolding of Truncated GROß Dimer*

The insoluble truncated groß [amino acids 5-73 of SEQ ID NO:3] in lysate pellet was solubilized in 1.3 liters of buffer containing 50 mm Tris HCl pH 8.0, 2 M guanidine HCl, 20 mM DTT by stirring overnight at room temperature. Soluble truncated groß [amino acids 5-73 of SEQ ID NO:3] was recovered by centrifugation at 25,000 g, from which guanidine HCl and DTT were removed by exhaustic dialysis against 50 mM sodium citrate pH 6.0 containing 2 mM EDTA to obtain soluble and reduced form of truncated groß [amino acids 5-73 of SEQ ID NO:3]. Truncated groß solution was concentrated to 3 mg/ml (Anicon YM3 membrane) and raised to pH 8.5 with 0.5 M Trizma base. Air oxidation of truncated groß [amino acids 5-73 of SEQ ID NO:3] was performed by stirring overnight at room temperature. Formation of dimer was monitored by Vydac C18 (Nest) using 20-40% linear gradient of acetonitrile in 0.1% TFA for 30 min.

C. *Purification of Dimer*

When dimer formation reached maximum, the reoxidation solution was adjusted to pH 8.0 with 10% acetic acid and the dimer captured on Toyopearl SP-650 M equilibrated in 25 mM Tris HCl pH 8.0 (Buffer A). The column was washed with 4 liters buffer A, 2 liters 0.125 M NaCl in buffer A, and eluted with 4 liters of linear gradient of 0.125 - 0.5 M NaCl in buffer A. Flow rate was 40 ml/min. Truncated groß dimer was well separated from truncated groß and other oligomer form of truncated groß (SDS-PAGE). Fractions containing truncated groß dimer were combined, adjusted to pH 3.0 with 10% TFA solution and applied to Vydac C18 (2.1 x 25 cm) equilibrated with 0.1% TFA in 4% acetonitrile. Truncated groß dimer was eluted with linear gradient of 20-40% acetonitrile in 0.1% TFA for 30 min. Truncated groß dimer was eluted at ~30% acetonitrile. Fractions containing truncated groß dimer was pooled, lyophilized to remove acetonitrile, and dialyzed in Spectrapor 3K MWCO dialysis tubing against PBS.

D. *Yield*

Typical yield of truncated groß dimer was 0.15 mg/g of cells when refolding was performed at 0.1 mg/ml or 0.7 mg/g of cells when refolding at 3 mg/ml.

E. *Characterization*

The molecular weight of the truncated groß dimer as determined on nonreducing SDS-PAGE was approximately twice that of truncated groß. Upon reduction, both forms migrated to the same spot indicating that truncated groß dimer is a disulfide linked dimer. The molecular weight of truncated groß dimer, as determined by MALD-MS analysis was 15,069 Da (predicted 15,073 Da), while that of truncated groß dimer was

7,536 Da (predicted 7,537 Da). N-terminal sequencing of truncated groß dimer showed that 5-10% of the final products retained the initiatory Met. Disulfide pairing pattern of truncated groß dimer was the same as that of truncated groß (C5-C31, C7-C47) [amino acids 5-73 of SEQ ID NO:3], however, all pairings were intermolecular rather than intramolecular. Gel filtration analysis and ultracentrifugation sedimentation equilibrium studies in PBS (pH 7.0) showed that truncated groß dimer exhibited reversible assembly of octamer to hexadecamer at 0.25 mg/ml, while truncated groß [amino acids 5-73 of SEQ ID NO:3] was a nonconvalent dimer even at 20 mg/ml. Concentration of truncated groß dimer has been determined by quantitative amino acid analysis.

**Example 3 - Prophylactically Administered Truncated KC in *E. coli* Sepsis.**

The animals were dosed intraperitoneally with truncated KC [amino acids 5-72 of SEQ ID NO:1] at doses of 10, 33, 100 or 333 fg/kg 24 hours and 2 hours before infection. Control animals were dosed with dilution buffer on the same schedule. Starting two hours after infection the rats were treated twice daily with subcutaneous gentamicin. On day 0 the rats were implanted with an *E. coli* containing fibrin-thrombin clot. Starting two hours after infection the rats were treated with gentamicin twice daily. The rats prophylactically treated with truncated KC at 33 or 100 fg/kg followed by gentamicin treatment demonstrated significantly improved survival rates over the diluent treated control rat receiving gentamicin therapy alone.

**Results**

| Dose (fg/kg) | survival (alive/dead) |
|---|---|
| Control | 8 / 17 |
| 10 | 10 / 15 |
| 33 | 17 / 8 |
| 100 | 18 / 7 |
| 333 | 10 / 15 |

**Example 4 - Therapeutically Administered Truncated KC in *E. coli* sepsis.**

On day 0 the rats were implanted with an *E. coli* containing fibrin-thrombin clot. The animals were dosed intraperitoneally with truncated KC [amino acids 5-72 of SEQ ID NO:1] at doses of 33, 100, 333, or 1,000 fg/kg as a single injection 2 hours after infection. Control animals were dosed with dilution buffer on the same schedule. Starting two hours after infection the rats were treated twice daily with subcutaneous gentamicin. The rats

therapeutically treated with truncated KC at 100 or 333 fg/kg followed by gentamicin treatment demonstrated significantly improved survival rates over the diluent treated control rat receiving gentamicin therapy alone.

**Results**

| Dose (fg/kg) | survival (alive / dead) |
|---|---|
| Control | 9 / 16 |
| 33 | 11 / 14 |
| 100 | 17 / 8 |
| 333 | 18 / 7 |
| 1,000 | 10 / 15 |

**Example 5 - Therapeutically Administered Truncated KC in *S. aureus* Sepsis.**

On day 0 the rats were implanted with a *S. aureus* containing fibrin-thrombin clot. The animals were dosed intraperitoneally with truncated KC [amino acids 5-72 of SEQ ID NO:1] at doses of 33, 100, 333, or 1,000 fg/kg as a single injection 2 hours after infection. Control animals were dosed with dilution buffer on the same schedule. Starting two hours after infection the rats were treated twice daily with subcutaneous gentamicin. The rats therapeutically treated with truncated KC at 100 or 333 fg/kg followed by gentamicin treatment demonstrated significantly improved survival rates over the diluent treated control rat receiving gentamicin therapy alone.

**Results**

| Dose (fg/kg) | survival (alive / dead) |
|---|---|
| Control | 8 / 17 |
| 33 | 11 / 14 |
| 100 | 17 / 8 |
| 333 | 21 / 4 |
| 1000 | 12 / 13 |

**Example 6 - Therapeutically Administered Truncated groß in *E. coli* Sepsis.**

On day 0 the rats were implanted with an *E. coli* containing fibrin-thrombin clot. The animals were dosed intraperitoneally with truncated groß [amino acids 5-73 of SEQ ID NO:3] at doses of 33, 100, 333, or 1,000 fg/kg as a single injection 2 hours after infection. Control animals were dosed with dilution buffer on the same schedule. Starting two hours after infection the rats were treated twice daily with subcutaneous gentamicin. The rats therapeutically treated with truncated groß at 100 or 333 fg/kg followed by gentamicin

treatment demonstrated significantly improved survival rates over the diluent treated control rats receiving gentamicin therapy alone.

**Results**

| Dose (fg/kg) | survival (alive/dead) |
|---|---|
| Control | 9 / 16 |
| 33 | 12 / 13 |
| 100 | 20 / 5 |
| 333 | 18 / 7 |
| 1000 | 10 / 15 |

**Example 7 - Therapeutically Administered Truncated groß in *S. aureus* Sepsis.**

On day 0 the rats were implanted with an *S. aureus* containing fibrin-thrombin clot. The animals were dosed intraperitoneally with truncated groß [amino acids 5-73 of SEQ ID NO:3] at doses of 33, 100, 333, or 1,000 fg/kg as a single injection 2 hours after infection. Control animals were dosed with dilution buffer on the same schedule. Starting two hours after infection the rats were treated twice daily with subcutaneous gentamicin. The rats therapeutically treated with truncated groß at 100 or 333 fg/kg followed by gentamicin treatment demonstrated significantly improved survival rates over the diluent treated control rats receiving gentamicin therapy alone.

**Results**

| Dose (fg/kg) | survival |
|---|---|
| Control | 9 / 16 |
| 33 | 12 / 13 |
| 100 | 20 / 5 |
| 333 | 18 / 7 |
| 1,000 | 10 / 15 |

**Example 8 - Therapeutical Subcutaneously Administered Truncated groß in *E. coli* Sepsis.**

On day 0 the rats were implanted with an *E. coli* containing fibrin-thrombin clot. The animals were dosed subcutaneously with truncated groß [amino acids 5-73 of SEQ ID NO:3] at doses of 0.1, 0.3, 1.0, or 3.3 pg/kg as a single injection 2 hours after infection. Control animals were dosed with dilution buffer on the same schedule. Starting two hours

after infection the rats were treated twice daily with subcutaneous gentamicin. The rats therapeutically treated with truncated groß at 0.3 or 1.0 pg/kg followed by gentamicin treatment demonstrated significantly improved survival rates over the diluent treated control rats receiving gentamicin therapy alone.

**Results**

| Dose (pg/kg) | survival (alive/dead) |
|---|---|
| Control | 10 / 15 |
| 0.1 | 12 / 13 |
| 0.3 | 18 / 7 |
| 1.0 | 20 / 5 |
| 3.3 | 11 / 14 |

**Example 9 - Therapeutical Subcutaneously Administered Truncated groß in *S. aureus* Sepsis.**

On day 0 the rats were implanted with an *S. aureus* containing fibrin-thrombin clot. The animals were dosed subcutaneously with truncated groß [amino acids 5-73 of SEQ ID NO:3] at doses of 0.1, 0.3, 1.0, or 3.3 pg/kg as a single injection 2 hours after infection. Control animals were dosed with dilution buffer on the same schedule. Starting two hours after infection the rats were treated twice daily with subcutaneous gentamicin. The rats therapeutically treated with truncated groß at 0.3 or 1.0 pg/kg followed by gentamicin treatment demonstrated significantly improved survival rates over the diluent treated control rats receiving gentamicin therapy alone.

**Results**

| Dose (pg/kg) | survival (alive/dead) |
|---|---|
| Control | 8 / 17 |
| 0.1 | 13 / 12 |
| 0.3 | 18 / 7 |
| 1.0 | 20 / 5 |
| 3.3 | 12 / 13 |

**Example 10 - Prophylactically Administered GROß Dimer in *E. coli* Sepsis.**

The animals were dosed subcutaneously with dimer formed of two truncated groß proteins [amino acids 5-73 of SEQ ID NO:3] at doses of 0.1, 0.3, 1.0 or 3.3 pg/kg 24 hours

before infection. Control animals were doses with dilution buffer on the same schedule. On day 0 the rats were implanted with an *E. coli* containing fibrin-thrombin clot. Starting two hours after infection the rats were treated twice daily with subcutaneous gentamicin. The rats prophylactically treated with truncated groß dimer at 0.3 or 1.0 pg/kg followed by gentamicin treatment demonstrated significantly improved survival rates over the diluent treated control rats receiving gentamicin therapy alone.

**Results**

| Dose (pg/kg) | survival (alive/dead) |
|---|---|
| Control | 8 / 17 |
| 0.1 | 10 / 15 |
| 0.3 | 18 / 7 |
| 1.0 | 20 / 5 |
| 3.3 | 8 / 17 |

Example 11 - **Therapeutically Administered GROß Dimer in *S. aureus* Sepsis.**

On day 0 the rats were implanted with an *S. aureus* containing fibrin-thrombin clot. The animals were dosed subcutaneously with a dimer formed of two truncated groß proteins [amino acids 5-73 of SEQ ID NO:3] at doses of 0.03, 0.1, 0.3, 1.0, 3.3, or 10 pg/kg as a single injection 2 hours after infection. Control animals were doses with dilution buffer on the same schedule.

Starting two hours after infection the rats were treated twice daily with subcutaneous gentamicin. The rats therapeutically treated with truncated groß dimer at 0.1, 0.3 or 1.0 pg/kg followed by gentamicin treatment demonstrated significantly improved survival rates over the diluent treated control rats receiving gentamicin therapy alone.

**Results**

| Dose (pg/kg) | survival (alive/dead) |
|---|---|
| Control | 11 / 14 |
| 0.03 | 12 / 13 |
| 0.1 | 18 / 7 |
| 0.3 | 23 / 2 |
| 1.0 | 24 / 1 |
| 3.3 | 17 / 8 |
| 10.0 | 12 / 13 |

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SmithKline Beecham Corporation
DeMarsh, Peter L.
Johanson, Kyung O.

(ii) TITLE OF INVENTION: Method of Treating Sepsis

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: SmithKline Beecham Corporation - Corporate Patents
(B) STREET: 709 Swedeland Road
(C) CITY: King of Prussia
(D) STATE: PA
(E) COUNTRY: USA
(F) ZIP: 19406-2799

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: WO
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/007,425
(B) FILING DATE: 21-NOV-1995

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Hall, Linda E.
(B) REGISTRATION NUMBER: 31,763
(C) REFERENCE/DOCKET NUMBER: P50417-1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 610-270-5016
(B) TELEFAX: 610-270-5090

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ala Pro Ile Ala Asn Glu Leu Arg Cys Gln Cys Leu Gln Thr Met
1 5 10 15

Ala Gly Ile His Leu Lys Asn Ile Gln Ser Leu Lys Val Leu Pro
20 25 30

Ser Gly Pro His Cys Thr Gln Thr Glu Val Ile Ala Thr Leu Lys
35 40 45

Asn Gly Arg Glu Ala Cys Leu Asp Pro Glu Ala Pro Leu Val Gln
50 55 60

Lys Ile Val Gln Lys Met Leu Lys Gly Val Pro Lys
65 70

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Ser Val Ala Thr Glu Leu Arg Cys Gln Cys Leu Gln Thr Leu
1 5 10 15

Gln Gly Ile His Pro Lys Asn Ile Gln Ser Val Asn Val Lys Ser
20 25 30

Pro Gly Pro His Cys Ala Gln Thr Glu Val Ile Ala Thr Leu Lys
35 40 45

Asn Gly Arg Lys Ala Cys Leu Asn Pro Ala Ser Pro Ile Val Lys
50 55 60

Lys Ile Ile Glu Lys Met Leu Asn Ser Asp Lys Ser Asn
65 70

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Pro Leu Ala Thr Glu Leu Arg Cys Gln Cys Leu Gln Thr Leu
1 5 10 15

Gln Gly Ile His Leu Lys Asn Ile Gln Ser Val Lys Val Lys Ser
20 25 30

Pro Gly Pro His Cys Ala Gln Thr Glu Val Ile Ala Thr Leu Lys
35 40 45

Asn Gly Gln Lys Ala Cys Leu Asn Pro Ala Ser Pro Met Val Lys
50 55 60

Lys Ile Ile Glu Lys Met Leu Lys Asn Gly Lys Ser Asn
65 70

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Ser Val Val Thr Glu Leu Arg Cys Gln Cys Leu Gln Thr Leu
1 5 10 15

Gln Gly Ile His Leu Lys Asn Ile Gln Ser Val Asn Val Arg Ser
20 25 30

Pro Gly Pro His Cys Ala Gln Thr Glu Val Ile Ala Thr Leu Lys
35 40 45

Asn Gly Lys Lys Ala Cys Leu Asn Pro Ala Ser Pro Met Val Gln
50 55 60

Lys Ile Ile Glu Lys Ile Leu Asn Lys Gly Ser Thr Asn
65 70

We Claim:

1. A method of treating sepsis comprising administering to an animal in need thereof an effective amount of a protein derived from a chemokine selected from the group consisting of (a) KC SEQ ID NO: 1, (b) groα SEQ ID NO: 2, (c) groß SEQ ID NO:3, and (d) groγ SEQ ID NO:4.

2. The method according to claim 1 wherein the chemokine is selected from the group consisting of:

(a) mature groß;

(b) modified groß consisting of amino acids 5 to 73 of SEQ ID NO: 3;

(c) a multimeric chemokine protein which comprises an association of two or more of (a) or (b); and

(d) a multimeric chemokine protein which comprises an association of (a) or (b) with a second chemokine.

3. The method according to claim 2 wherein the chemokine is a dimeric protein consisting of two modified groß proteins.

4. The method according to claim 1 wherein said chemokine is selected from the group consisting of:

(a) mature groα;

(b) modified groα consisting of amino acids 5 to 73 of SEQ ID NO: 2;

(c) a multimeric chemokine protein which comprises an association of two or more of (a) or (b); and

(d) a multimeric chemokine protein which comprises an association of (a) or (b) with a second chemokine.

5. The method according to claim 1 wherein said chemokine is selected from the group consisting of:

(a) mature groγ;

(b) modified groγ consisting of amino acids 5 to 73 of SEQ ID NO: 4;

(c) a multimeric chemokine protein which comprises an association of two or more of (a) or (b); and

(d) a multimeric chemokine protein which comprises an association of (a) or (b) with a second chemokine.

6. The method according to claim 1 wherein said chemokine is selected from the group consisting of:

(a) mature KC;

(b) modified KC consisting of amino acids 5 to 72 of SEQ ID NO: 1;

(c) a multimeric chemokine protein which comprises an association of two or more of (a) or (b); and

(d) a multimeric chemokine protein which comprises an association of (a) or (b) with a second chemokine.

7. A method according to claim 1 wherein said effective amount is from about 10 to about 1,000 fg/kg/dose.

8. The method according to claim 1 wherein said chemokine is administered 2 hours to 24 hours after surgery.

9. The method according to claim 1 wherein said chemokine is administered orally.

10. The method according to claim 1 wherein said chemokine is administered subcutaneously.

11. The method according to claim 1 further comprising the step of administering the chemokine in conjunction with an effective amount of an anti-infective agent.

12. A method according to claim 11 wherein the anti-infective agent is selected from the group consisting of gentamicin, augmentin or ceftazidime.

13. A method for the prevention of sepsis comprising administering to an animal in need thereof an effective amount of an effective amount of a protein derived from a chemokine selected from the group consisting of (a) KC SEQ ID NO: 1, (b) groα SEQ ID NO: 2, (c) groß SEQ ID NO:3, and (d) groγ SEQ ID NO:4.

14. The method according to claim 13 wherein the chemokine is selected from the group consisting of:

(a) mature groß;

(b) modified groß consisting of amino acids 5 to 73 of SEQ ID NO: 3;

(c) a multimeric chemokine protein which comprises an association of two or more of (a) or (b); and

(d) a multimeric chemokine protein which comprises an association of (a) or (b) with a second chemokine.

15. The method according to claim 14 wherein the chemokine is a dimeric protein consisting of two modified groß proteins.

16. The method according to claim 13 wherein said chemokine is selected from the group consisting of:

(a) mature groα;

(b) modified groα consisting of amino acids 5 to 73 of SEQ ID NO: 2;

(c) a multimeric chemokine protein which comprises an association of two or more of (a) or (b); and

(d) a multimeric chemokine protein which comprises an association of (a) or (b) with a second chemokine.

17. The method according to claim 13 wherein said chemokine is selected from the group consisting of:

(a) mature groγ;

(b) modified groγ consisting of amino acids 5 to 73 of SEQ ID NO: 4;

(c) a multimeric chemokine protein which comprises an association of two or more of (a) or (b); and

(d) a multimeric chemokine protein which comprises an association of (a) or (b) with a second chemokine.

18. The method according to claim 13 wherein said chemokine is selected from the group consisting of:

(a) mature KC;

(b) modified KC consisting of amino acids 5 to 72 of SEQ ID NO: 1;

(c) a multimeric chemokine protein which comprises an association of two or more of (a) or (b); and

(d) a multimeric chemokine protein which comprises an association of (a) or (b) with a second chemokine.

19. A method according to claim 13 wherein the effective amount is from about 10 to about 1,000 fg/kg/dose.

20. The method according to claim 13 wherein said chemokine is administered 1 to 2 days prior to surgery.

21. The method according to claim 13 further comprising the step of administering the chemokine in conjunction with an effective amount of an anti-infective agent.

22. A method according to claim 21 wherein the anti-infective agent is selected from the group consisting of gentamicin, augmentin or ceftazidime.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/18616

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C12N 15/19; A61K 38/19
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.1; 435/69.5, 71.1, 71.2, 172.3, 252.3, 320.1; 514/2, 8, 12; 530/300,324

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | ARTURSON, G. Neutrophil granulocyte functions in severely burned patients. Burns. 1985, Vol. 11, pages 309-319. | 1-22 |
| A | JANSEN et al. Monocyte Chemotactic Protein 1 is Released during Lethal and Sublethal Bacteremia in Baboons. The Journal of Infectious Diseases. June 1995, Vol. 171, pages 1640-1642. | 1-22 |
| A | BOSSINK et al. Plasma Levels of the Chemokines Monocyte Chemotactic Protein-1 and -2 Are Elevated in Human Sepsis. Blood. 15 November 1995, Vol. 86, No. 10, pages 3841-3847. | 1-22 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>13 FEBRUARY 1997 | Date of mailing of the international search report<br>11 MAR 1997 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>PREMA MERTZ<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/18616

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | DRISCOLL, K.E. Macrophage Inflammatory Proteins: Biology and Role in Pulmonary Inflammation. Experimental Lung Research. 1994, Vol. 20, pages 473-490. | 1-22 |
| A | BURGMANN et al. Serum Concentrations of MIP-1 $\alpha$ and Interleukin-8 in Patients Suffering from Acute *Plasmodium falciparum* Malaria. Clinical Immunology and Immunopathology. July 1995, Vol. 76, No. 1, pages 32-36. | 1-22 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/18616

A. CLASSIFICATION OF SUBJECT MATTER:
US CL. :

424/85.1; 435/69.5, 71.1, 71.2, 172.3, 252.3, 320.1; 514/2, 8, 12; 530/300,324

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, MEDLINE, BIOSIS
search terms: chemokine, KC, gro-alpha or melanoma growth stimulating factor, gro-beta or macrophage inflammatory protein-2 alpha or MIP-2 alpha, gro-gamma or macrophage inflammatory protein-2 beta or MIP-2 beta, administration or treatment or therapy.